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Induction of Adenine Salvage in Mouse Cell Lines Deficient in Adenine Phosphoribosyltransferase

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Adenine phosphoribosyltransferase (APRT) (EC 2.4.2.7) pseudorevertant cell lines were isolated under selective conditions requiring adenine salvage for survival; yet they were found to be deficient in measurable APRT activity and resistant to the purine analog 2′6′-diaminopurine (DAP) (M. S. Turker, J. A. Tischfield, P. Rabinovitch, P. J. Stambrook, J. J. Trill, A. C. Smith, C. E. Ogburn, and G. M. Martin, manuscript in preparation). Adenine salvage was examined in two APRT pseudorevertant cell lines, their two APRT homozygous deficient parental cell lines, and a genotypic APRT revertant cell line (i.e., one with measurable APRT activity and DAP sensitivity). Adenine accumulation was observed in both revertant phenotypes and was demonstrated by high-performance liquid chromotography to be linked with adenine metabolism. The ability to salvage adenine declined substantially in the pseudorevertant cell lines when they were removed from selective media containing inhibitors of de novo 5′-AMP synthesis (alanosine and azaserine); for one pseudorevertant cell line this decline was accelerated by the addition of DAP to the medium. The readdition of alanosine or azaserine to the growth medium of the pseudorevertant lines induced adenine salvage to its previous levels. An APRT-like cross-reacting material was found in the pseudorevertant cell lines, although its relationship to adenine salvage is unknown. A low level of constitutive adenine salvage was found in the parental APRT-deficient lines, and it was also possible to induce adenine salvage in these cell lines. These findings suggest a novel regulatory mechanism for adenine salvage.

It has been demonstrated by fluctuation analysis (15) and replica plating (12) that in bacteria, mutations occur spontaneously in the absence of selective pressure. These observations have served as the basis for mutational analysis at many mammalian loci, using various selective systems that identify but do not induce mutant and revertant genotypes (5, 7, 24). The toxic purine analog 2′6′-diaminopurine (DAP) is routinely used for the selection of adenine phosphoribosyltransferase (APRT)-deficient cell lines, and it has been demonstrated that such cells can be identified (1) and selected (2) in the absence of DAP-containing medium. APRT catalyzes the conversion of adenine to 5′-AMP (16), and it is the enzyme responsible for the accumulation of adenine against a concentration gradient (i.e., adenine salvage) (3, 26). It is therefore possible to select for reversion at the APRT locus by blocking de novo synthesis of 5′-IMP with azaserine (7) or the conversion of 5′-IMP to 5′-AMP with alanosine (5) and forcing cells to salvage adenine for survival. Reversion at the APRT locus is usually linked to reacquisition of enzymatic activity (4, 25). Although the molecular bases underlying forward mutations at the APRT locus have been partially elucidated (20, 22, 23), detailed studies concerning the nature of reversion at this locus are still lacking.

In other work, we will describe the isolation of differentiated mouse cell lines selected from homozygous APRT-deficient parentals for their ability to salvage adenine. Suprisingly, most of these lines were deficient in detectable APRT activity and are therefore termed APRT pseudorevertants (M. S. Turker, J. A. Tischfield, P. Rabinovitch, P. J. Stambrook, J. J. Trill, A. C. Smith, C. E. Ogburn, and G. M. Martin, manuscript in preparation). They were also found to be DAP resistant (DAP^r), consistent with their apparent APRT deficiency. In this study, we demonstrate that pseudorevertant cell lines can accumulate and metabolize adenine, indicating active adenine salvage. The ability to salvage adenine is normally expressed at a low constitutive level in homozygous APRT-deficient cells and it is readily induced to high levels by exposure to alanosine or azaserine. We propose that a novel regulatory mechanism for adenine salvage is expressed in the pseudorevertant cell lines.

MATERIALS AND METHODS

Cell strains. Isolation, initial characterization, and growth media for the cell strains used in this study will be described in a subsequent publication (Turker et al., in preparation). The following strains (abbreviations are given in parentheses) were used: TC-DAPlC-21 (TC-21), TC-DAPlC-21R10 (TC-21R10), TT-DAPlC-17 (TT-17), TT-DAPlC-17R21 (TT-17R21) and TT-E140-3D3R10 (TT-3D3R10). An APRT-deficient L-cell line was obtained from Stanley Gartler, Department of Genetics, University of Washington, Seattle. TC-21R10 and TT-17R21 are APRT pseudorevertant lines clonally derived from the APRT homozygous deficient lines TC-21 and TT-17, respectively. TT-3D3R10 is a genotypic APRT revertant cell line.

Adenine accumulation. This assay is similar to that previously reported by Witney and Taylor (26). Nearly confluent or confluent cells in a flask (25 cm^2) were exposed to 3 ml of medium containing 6 μM [^{14}C]adenine (8.3 Ci/mol; Amersham Corp., Arlington Heights, Ill.) at 37°C. Other temperatures used are indicated below. An adenine concentration of 6 μM was selected because at this concentration, adenine phosphorylation is favored over the accumulation of free adenine (3). The cells were incubated with the labeled precursor for 5 to 60 min and then washed three times with ice-cold phosphate-buffered saline (pH 7.4). Care was taken to remove as much phosphate-buffered saline as possible

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after the third wash; as a result, approximately 0.1 ml of phosphate-buffered saline was left in each 25-cm^2 flask. The cells were than dissolved in 1 ml of 0.2 N NaOH for 20 h at 37°C. Portions of each sample (200 μl) were then counted by liquid scintillation in Aquasol II (New England Nuclear Corp., Boston, Mass.), and 25-μl aliquots were used to determine the protein amount (14). Accumulation was expressed as nanomoles of adenine per milligram of protein per unit of time. The background counts per 200-μl sample were less than 100 cpm for a 25-cm^2 flask without cells. The extent of adenine accumulation was assumed to include adenine binding, free intracellular adenine, and metabolized adenine. Cell lines maintained in medium containing adenine (60 μM) and either the antibiotic alanosine (20 μM; from J. Tischfield, Department of Anatomy, Georgia Medical College) or azaserine (50 μM; Sigma Chemical Co., St. Louis, Mo.) were placed in nonselective medium supplemented with 10% dialyzed fetal bovine serum (DFBS) 24 h before being assayed. This was necessary because the cells were grown in medium containing 60 μM adenine but were assayed in medium containing 6 μM adenine. Therefore, time was allowed for the intracellular concentration of free adenine to fall. Cells maintained in the presence of 72 μg of DAP (Sigma) per ml were placed in medium still containing DAP but with 10% DFBS 24 h before being assayed. These cells were placed in Dulbecco minimal essential medium (DMEM) and DFBS without DAP 10 min before being assayed.

Preparation of acid-soluble and acid-insoluble cellular fractions. Flasks (25 cm^2) containing confluent or nearly confluent cell cultures were incubated with [^{14}C]adenine under the conditions described above. After the phosphate-buffered saline wash, 4 ml of ice-cold 10% trichloroacetic acid (TCA) was added to each flask, and incubation was continued at 4°C for 1 h. The TCA solution (acid-soluble fraction) was decanted from the flask and extracted with ether five times, passed over a disposable C_{18} cartridge (Sep-Pak; Waters Associates, Milford, Mass.), lyophilized, and suspended in 100 to 200 μl of twice-distilled H_2O. The acid-insoluble fraction (the fixed cells on the flask surface) was washed three times with 5% TCA and dissolved in 1 ml of 0.3 N KOH by incubation at 37°C for 20 h. This solution was then neutralized with 6 N perchloric acid, and the resultant precipitate was removed by centrifugation with an Eppendorf microfuge. The supernatant was then mixed with ice-cold 20% TCA (to remove DNA), incubated at 4°C for 1 h, and centrifuged again. The TCA supernatant, containing products of RNA hydrolysis, was then treated as above for the acid-soluble fraction.

Reverse-phase HPLC analysis of nucleotides and free purine bases. Reverse-phase high-pressure liquid chromatography (HPLC) was performed on a Waters Associates liquid chromatograph equipped with a model 680 autogradient controller, model 510 pumps, model U6k injector, and a model 441 absorbance detector. A C_{18} reverse-phase column (4.6 mm [inner diameter] by 25 cm) (Ultrasphere-ODS; Beckman Instruments, Palo Alto, Calif.) was used.

The acid-soluble fractions (20 to 90 μl) were mixed with 5-μl solutions containing authentic purine bases and nucleotides (1 mg/ml for each standard marker), applied to the column, and eluted at a flow rate of 1 ml/min. Two solutions were used to elute the purine bases and nucleotides: 0.2 M ammonium phosphate (pH 5.1) (solution A) and 60% methanol (solution B). A linear gradient was established, beginning with 100% solution A at 0 min and ending with 50% solution A and 50% solution B at 25 min. This mixture was maintained for an additional 5 min. Standard markers were recorded as A_{254}, and 0.5-min fractions were collected and counted by liquid scintillation. The RNA extracts were treated as above, except that the following linear gradient was established: 0 min, 100% solution A; 26 min, 78% solution A–22% solution B. This mixture was maintained for an additional 14 min. This method facilitated the separation of 3′-AMP and 2′-GMP.

Detection of APRT-like CRM with rabbit anti-mouse APRT antibody. The competition assay used for cross-reacting material (CRM) had been previously described (25) and is a modification of the method of Simon et al. (23). Briefly, a cell extract lacking measurable APRT activity was mixed with a cell extract containing wild-type mouse APRT enzyme. The teratocarcinoma cell line PSA-1-76 (25) was used as a source of wild-type enzyme. This mixture was then combined with rabbit anti-mouse APRT antibody at dilutions ranging from 1:8 to 1:1,024 for 2 h. Free antibody and antibody-antigen complexes were precipitated with Pansorbin (Calbiochem-Behring, La Jolla, Calif.), and the APRT activity remaining in the supernatant was then assayed.

Mycoplasma detection. Mycoplasma detection was performed as described by Russell et al. (21) and as described by McGarrity and Carson (17).

Cytogenetics. Preparation of metaphase spreads was performed as described by Turker et al. (25).

RESULTS

Adenine salvage. Adenine accumulation increased with time at 37°C in the APRT pseudorevertant cell lines TT-17R21 and TC-21R10 and in a genotypic APRT revertant line TT-3D3R10 (Fig. 1A). The rates of accumulation increased linearly with time for 20 min, and thereafter, all three plots became biphasic, with lesser rates of accumulation. Markedly reduced yet apparently significant amounts of adenine accumulation were seen in the homozygous APRT-deficient cell lines TC-21 and TT-17 but not in an APRT-deficient mouse L-cell line (Fig. 1B). Adenine accumulation was also seen at room temperature (20 to 21°C) in all lines except the L-cell line but was markedly diminished at 4°C (data not shown).

Acid-soluble cellular fractions were analyzed by HPLC to determine whether adenine metabolism was responsible for its accumulation within the cells. Cells were incubated in the presence of [^{14}C]adenine at 37°C for various times from 2 min to 4 h. Table 1 shows the results obtained for the 2-min incubation for the pseudorevertant cell lines TC-21R10 and TT-17R21 and their homozygous deficient parental lines TC-21 and TT-17, respectively. At this short time, adenine metabolism was evidenced by a radiolabeled peak with a retention time identical to that of 5′-IMP and ATP (these two standards had the same retention time). Greater than 90% of the radioactivity in the pseudorevertant fractions was due to 5′-IMP or ATP; the remainder was due to free adenine. In contrast, greater than 85% of the radioactivity found in the homozygous deficient parental fractions was in the form of free adenine. It is significant to note that the remainder of the radioactivity was either 5′-IMP or ATP, thereby confirming adenine metabolism in these cells. The 5′-IMP–ATP peak rose to greater that 50% in the homozygous deficient cells incubated with [^{14}C]adenine for 30 min (Table 1). Fractions obtained from the pseudorevertant cell lines and the genotypic revertant line, TT-3D3R10, when incubated with the [^{14}C]adenine for 20 to 30 min, revealed many radiolabeled peaks. These included a peak comigrating with authentic 5′-AMP, the product of APRT activity (data not shown). We

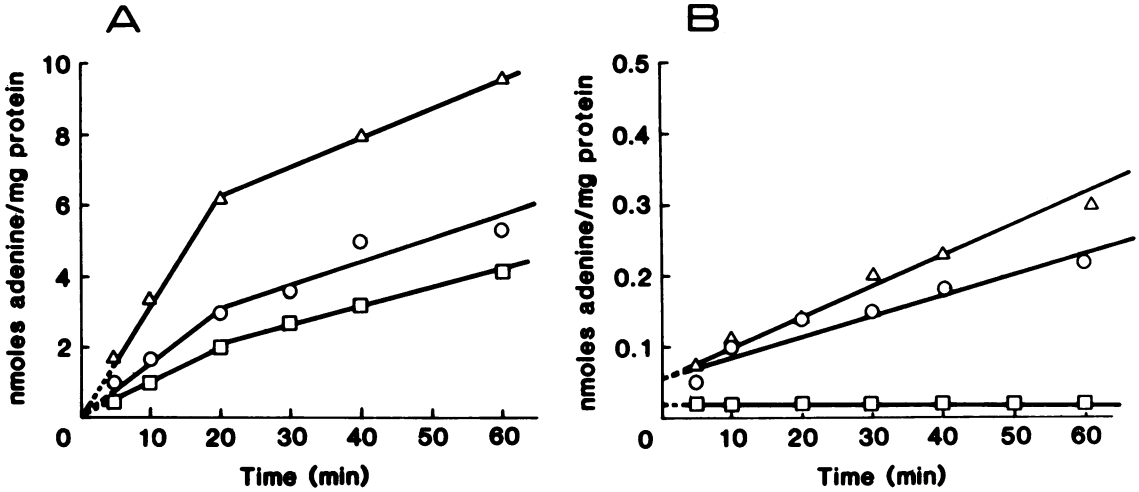

FIG. 1. (A) Adenine accumulation at 37°C in APRT pseudorevertants TC-21R10 (□) and TT-17R21 (○) and genotypic APRT revertant TT-3D3R10 (△). (B) Adenine accumulation at 37°C in parental homozygous deficient lines TC-21 (△) and TT-17 (○) and APRT-deficient L cells (□).

never observed a radiolabeled peak comigrating with adenosine, the product of mycoplasmal adenosine phosphorylase activity, even in the acid-soluble fractions incubated with [^{14}C]adenine for 4 h.

Adenine metabolism was also seen at room temperature (20 to 21°C) for the genotypic revertant cell line and the one pseudorevertant cell line tested, TC-21R10. Both revertant phenotypes were incubated with [^{14}C]adenine for 2 min (Table 1). When these two cell lines were maintained nonselectively (i.e., without adenine and azaserine in the medium) for 5 days and then assayed for the ability to metabolize adenine within two minutes at room temperature, a significant difference was observed. The genotypic revertant was still able to metabolize adenine, whereas the pseudorevertant cell line was unable to convert adenine to any metabolic product(s) under this condition (Table 1). This

TABLE 1. HPLC analysis of adenine accumulation at 37°C and room temperature

Temp, cell line	Phenotype[a]	Time (min)[b]	Free adenine		IMP-ATP	
			cpm	%	cpm	%
37°C						
TC-21	HD	2	1,417	91.8	127	8.2
TC-21	HD	30	1,344	41.3	1,913[c]	58.7
TC-21R10	PR	2	58	1.7	3,465	98.3
TT-17	HD	2	623	85.0	110	15.0
TT-17	HD	30	698	23.3	2,295[c]	76.7
TT-17R21	PR	2	99	7.5	1,223	92.5
Room temp[d]						
TC-21R10	PR	2	411	60.3	271	39.7
TT-3D3R10	GR	2	299	12.5	2,098	87.5
TC-21R10[e]	PR	2	4,407	100.0	0	0.0
TC-3D3R10[e]	GR	2	856	42.5	1,156	57.5

[a] HD, APRT homozygous deficient; PR, APRT pseudorevertant; GR, APRT genotypic revertant.
[b] Incubation time in the presence of [^{14}C]adenine.
[c] These numbers include approximately 250 cpm from a radiolabeled peak tentatively identified as 5'-GMP.
[d] 20 to 21°C.
[e] These cells were maintained nonselectively for 5 days before incubation at room temperature with [^{14}C]adenine.

result indicated that adenine salvage was unstable in the pseudorevertant cell line when it was maintained nonselectively.

To establish that salvaged adenine was being incorporated into macromolecules in the pseudorevertant cell line TC-21R10, we looked for incorporation of [^{14}C]adenine into macromolecular RNA. This was also done for the genotypic revertant line TT-3D3R10. Both lines were incubated with medium containing [^{14}C]adenine for 2 and 4 h at 37°C, and the TCA-insoluble fractions were dissolved with KOH to generate 2' and 3' monophosphates from RNA. Incorporation of salvaged adenine into RNA was found for both revertant cell types by this method (data not shown).

Adenine salvage decreases in nonselective and DAP media. To further test for the possibility that adenine salvage was unstable in the pseudorevertant cell lines, adenine accumulation was assayed for 30 min at 37°C for the pseudorevertant cell lines TT-17R21 and TC-21R10 grown nonselectively and in the presence of DAP for 24 to 96 h. Growth in DAP for 24 h slightly reduced the amount of adenine accumulation (Table 2), except in one case, in which the reduction was quite severe. By 48 h, however, adenine accumulation was reduced in both cell lines to the low levels seen in their homozygous deficient parental cell lines TT-17 and TC-21. Nonselective maintenance for 24 to 96 h also led to reduction in adenine salvage capabilities. It should be noted that removal of cultures from azaserine or alanosine media for 24 h was the normal assay method used herein. For TT-17R21, 48 h of nonselective incubation was sufficient to decrease the adenine salvage of this cell line to a basal constitutive level, whereas salvage activity for TC-21R10 was not diminished as rapidly. Therefore, at least for the pseudorevertant line TC-21R10, exposure to DAP medium resulted in the diminishment of adenine salvage at a faster rate than that resulting from transfer to nonselective medium (Table 2).

Adenine salvage is induced by exposure to alanosine and azaserine. The pseudorevertant cell lines TT-17R21 and TC-21R10 were grown in DAP medium for 5 to 7 days and moved to medium containing adenine and alanosine, adenine and azaserine, or adenine alone for 48 to 96 h. The cells were subsequently assayed for adenine accumulation after an additional 24 h of incubation in nonselective medium (see Materials and Methods). Exposure for 48 h to either antibi-

TABLE 2. The effect of DAP and removal from selective medium upon adenine accumulation in the pseudorevertant cell lines

Cell line	Adenine accumulation in[a]:							
	Cells maintained in DAP for[b]:				Cells maintained nonselectively for[c]:			
	24 h	48 h	72 h	96 h	24 h[d]	48 h	72 h	96 h
TC-21R10	0.29	0.22	0.22	—	1.58	0.95	0.59	—
TC-21R10	—	0.13	0.13	0.15	2.60	—	—	1.05
TC-21R10	2.34	0.44	—	—	2.95	1.53	1.66	—
TT-17R21	2.00	0.06	0.06	—	3.09	0.05	0.05	—
TT-17R21	—	0.06	0.04	0.05	1.14	—	—	0.07
TT-17R21	3.00	0.12	—	—	3.84	0.12	0.12	—

[a] Cells were exposed to [^{14}C]adenine for 30 min at 37°C. Results are expressed as nanomoles of adenine accumulated per milligram of protein. Each line represents an individual experiment. —, Not tested.

[b] Cells were maintained in 72 μg of DAP per ml for 24 to 96 h as indicated. They were removed from DAP medium 10 min before being exposed to adenine-containing medium.

[c] Cells were removed from revertant selective media and maintained nonselectively for 24 to 96 h as indicated. They were then assayed for adenine accumulation.

[d] Adenine accumulation was routinely measured 24 h after the cultures were removed from selective media (see Materials and Methods).

otic was sufficient to induce the pseudorevertant cell lines to express adenine salvage at their respective original levels. Adenine alone in the medium did not have this effect. The homozygous deficient lines TC-21 and TT-17 were tested in this manner, and they were also found to possess an inducible adenine salvage mechanism (Table 3).

An APRT-like CRM is present in the pseudorevertant cell lines. Rabbit anti-mouse APRT antibody was used in a competition assay to search for APRT-like CRM in cell extracts from the pseudorevertant cell lines TT-17R21 and TC-21R10. Figure 2 shows the result for TC-21R10 and demonstrates a significant level of CRM in this cell line. A similar, although not as dramatic, result was obtained for TT-17R21 with an extract containing only one-half the amount of cellular protein. CRM was also detected in an extract from the genotypic revertant TT-3D3R10. In this case, the enzyme was heat inactivated (25), allowing the APRT molecules to retain some antigenicity without any detectable enzymatic activity. A lesser amount of CRM was detected in extracts from the homozygous deficient lines TC-21 (Fig. 2) and TT-17. We also analyzed cell extracts from the pseudorevertant cell lines after growth in the presence of DAP for 7 days and found a greatly reduced amount of CRM as compared with these same cells grown in APRT reversion media (Fig. 2, TC-21R10). It should also be noted that this assay required the mixing of pseudorevertant extracts (containing no detectable APRT activity) with a wild-type extract (containing APRT activity). This mixing was done at approximately a 10:1 protein ratio, and it did not alter the amount of wild-type activity observed. This observation argues against an APRT inhibitor or significant phosphatase activity being present in the pseudorevertant extracts.

Chromosomal analysis. Metaphase spreads were prepared from the pseudorevertant cell lines and their homozygous deficient parental lines for chromosomal analysis. This examination did not reveal any homogeneous staining regions within chromosomes, extrachromosomal minute or double minute fragments, or translocations involving chromosome no. 8. This chromosome carries the APRT locus (11).

Mycoplasma detection. To ensure that adenine salvage was not due to mycoplasmal contamination (10), two detection methods were used. The first method was based on the visualization of mycoplasmal DNA with a fluorescent dye (21). All five lines used for this study were found to be mycoplasma free by this procedure. The second method was based on toxicity mediated by adenosine phosphorylase, an enzyme present in mycoplasmal species but not in mammalian cells (17). Mycoplasma-infected cells die rapidly when grown in the presence of 6-methyl-(purine deoxyriboside), whereas noninfected cells grow normally. Both pseudorevertant cell lines were tested in 1 and 10 μM 6-methyl-(purine deoxyriboside) while in the induced state (i.e., grown in the presence of antibiotic and adenine), and both were found to grow at a normal rate.

DISCUSSION

In a previous study, we isolated multipotent mouse teratocarcinoma stem cells exhibiting unstable expression for APRT activity and provided evidence against a classical mutation as the underlying cause for this high-frequency switching event (25). We next asked if this switching event was expressible in more specialized (i.e., differentiated) cell types and found that the answer was yes, but with a wrinkle (Turker et al., in preparation). In the differentiated cells, high-frequency reversion for the ability to salvage adenine was found to be associated with a novel phenotype, termed pseudoreversion. Pseudorevertants were found to be DAP^r and yielded cell extracts severely deficient in APRT activity. We have demonstrated here that pseudorevertants do indeed salvage adenine at significant levels, that adenine salvage is induced and maintained by the selective media, and that an APRT-like CRM is present within these cells. These results imply a novel regulatory mechanism for adenine salvage in the cells we have studied.

Total cellular adenine accumulation was measured in two

TABLE 3. The effect of azaserine or alanosine on adenine accumulation in the pseudorevertant cell lines and their homozygous deficient parental cell lines

Cell line, antibiotic[a]	Adenine accumulation[b]				
	48 h	72 h	96 h	DAP[c]	Adenine[d]
TC-21R10					
Azaserine	2.15	—	2.33	0.14	0.13
Azaserine	1.92	2.04	—	0.17	0.15
TT-17R21					
Alanosine	1.95	—	—	0.07	0.05
Alanosine	3.27	3.46	—	0.17	0.15
TC-21					
Azaserine	2.22	1.64	—	0.07	—
Alanosine	2.07	—	—	—	—
TT-17					
Azaserine	2.62	3.57	—	0.08	—
Alanosine	0.36[e]	—	—	—	—

[a] 60 μM adenine with either 50 μM azaserine or 20 μM alanosine, as indicated.

[b] Cells were maintained in DAP and then shifted to the conditions indicated. They were assayed for adenine accumulation for 30 min at 37°C. Results are expressed as nanomoles of adenine accumulated per milligram of protein. Each line represents an individual experiment. —, Not tested.

[c] Cells grown continuously in DAP for at least 96 h.

[d] 60 μM adenine with no antibiotic added.

[e] These cells failed to grow during the 48 h of exposure to alanosine and adenine.

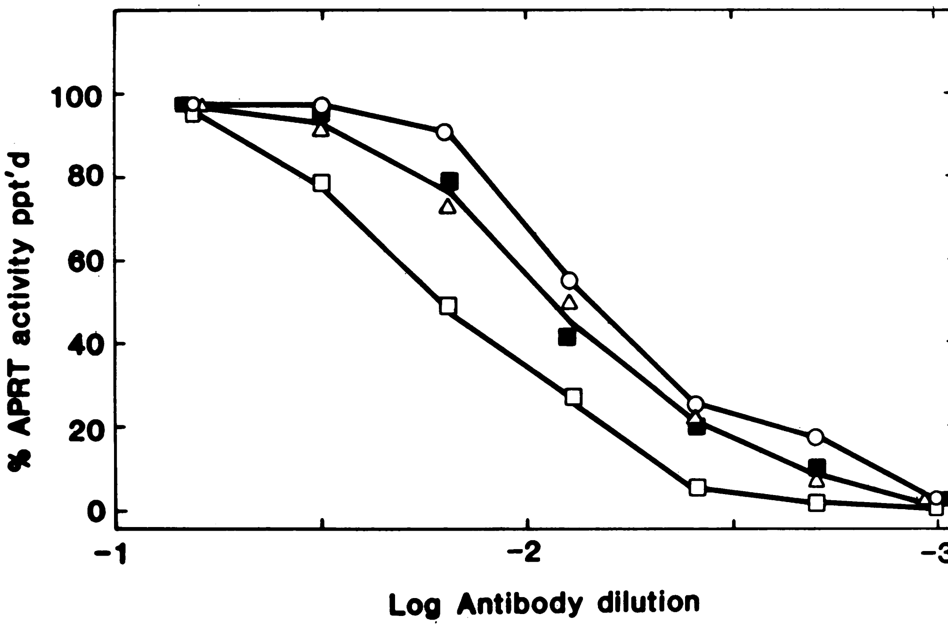

FIG. 2. Antibody dilution profiles for wild-type APRT enzyme activity mixed with Tris buffer (○) (mean of six assays) and cell extracts in Tris buffer from TC-21 in DAP medium (△; mean of two assays), TC-21R10 in adenine and azaserine medium (□; mean of three assays), and TC-21R10 in DAP medium (■; mean of two assays). PSA-1-76 cells (25) served as a source of wild-type APRT enzyme. TC-21 and TC-21R10 cell extracts contained identical protein amounts and were mixed at a 10:1 protein ratio with the PSA-1-76 cell extract.

pseudorevertant cell lines (TC-21R10 and TT-17R21) and in a genotypic revertant line (TT-3D3R10). The rate of adenine accumulation was found to occur at high levels in both types of revertants, being somewhat higher for TT-3D3R10. A low constitutive level of adenine salvage is apparently maintained in the homozygous APRT-deficient parental lines, TC-21 and TT-17. Although adenine accumulation occurs at a much slower rate in these cells than in the pseudorevertant lines derived from them, measurable accumulation occurring in these cells has been shown by HPLC to reflect metabolism (see below). Such accumulation was observed in experiments in which the cells were removed from DAP selection only 10 min before being incubated with [^{14}C]adenine. It could be diminished, however, at 4°C, as it was in the pseudorevertant cell lines. Therefore, it seems likely that the low level of adenine salvage seen in the parental cell lines TC-21 and TT-17 occurs via the same mechanism present in the pseudorevertant lines TC-21R10 and TT-17R21. This would suggest that the phenomenon of pseudoreversion is due to an increase in the expression of this adenine salvage mechanism rather than activation of a separate mechanism.

Increases in enzymatic functions are occasionally due to amplification of specific DNA regions. Evidence of such amplification is frequently manifested as homogeneous staining regions within chromosomes or extrachromosomal fragments known as minutes (6, 13). One report also mentioned the possibility that an increase in HPRT activity could result from chromosomal translocation involving the X chromosome (18). The changes in adenine salvage levels we have reported are unlikely to be due to gene amplification or chromosomal translocation for several reasons. Chromosomal analysis of the pseudorevertant cell lines failed to reveal the presence of homogeneous staining regions, minutes, or translocations involving chromosome no. 8, which carries the APRT locus (11). In studies of the HPRT locus, amplification of a mutant HPRT gene occasionally leads to phenotypic reversion. Since the mutant gene expresses a small amount of HPRT activity, overexpression of the mutant gene leads to an increase in this activity. Therefore, phenotypic reversion for hypoxanthine salvage is always accompanied by an increase in HPRT activity (9, 18, 27). The APRT pseudorevertants we have reported regain the ability to salvage adenine without a concomitant increase in APRT activity (Turker et al., in preparation). We also note that all evidence we have obtained to date indicates that the initial loss of APRT activity to produce homozygous deficient cells occurred via a nonclassical gene mutation (i.e., it did not affect the primary structure of the expressed APRT gene) (25; Turker et al., in preparation). Finally, amplification-associated reversion at the HPRT locus occurs at clonal frequencies, as does the loss of these amplified sequences. The phenotypic switching we have described for acquisition and loss of adenine salvage capability occurs rapidly (within 48 h) at the mass population level.

The first step in APRT-catalyzed adenine salvage is the addition of a phosphorylated ribose moiety to produce 5′-AMP (16, 19). 5′-AMP can then be further metabolized to produce a number of products, including ATP, 5′-IMP, and adenosine. Adenosine is formed from 5′-AMP in cells expressing 5′-nucleotidase (19). Our HPLC analysis of adenine salvage in the pseudorevertant cells, their homozygous deficient parentals, and the genotypic revertant line demonstrated clear evidence of adenine metabolism within 2 min at 37°C. This evidence was in the form of a radiolabeled peak with a retention time identical to 5′-IMP and ATP. 5′-AMP was seen in samples derived from the revertant cell types incubated with [^{14}C]adenine for 20 min or longer at 37°C. A small 5′-AMP peak was also seen in the genotypic revertant line when cells were incubated for 30 s at room temperature (data not shown). Therefore it is plausible that in the cell lines we have examined herein, adenine is converted to 5′-AMP and the resultant 5′-AMP rapidly undergoes further metabolism. Long incubation times would be sufficient to allow some unmetabolized 5′-AMP to accumulate. Other

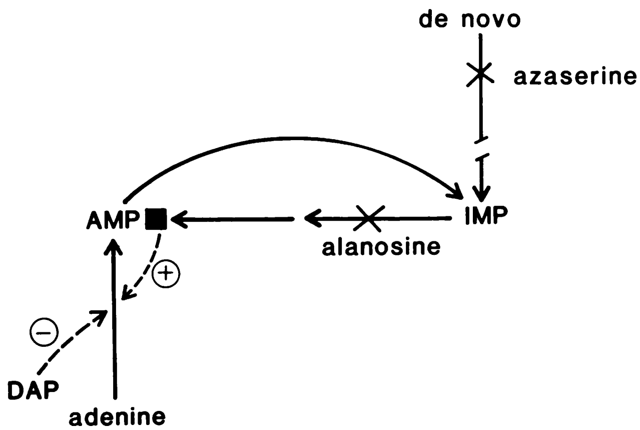

FIG. 3. Model showing the relationship between adenine salvage and 5'-AMP starvation in the pseudorevertant cell lines. This adenine salvage mechanism is normally expressed at a low constitutive level. When de novo 5'-AMP formation is blocked (■) with either azaserine or alanosine (×), adenine salvage is induced to high levels. DAP may have a negative effect on adenine salvage.

possibilities for adenine metabolism seem unlikely. The conversion of adenine directly to adenosine would require a purine nucleoside phosphorylase enzyme with a specificity for adenine, but such an enzyme has not been described for mammalian cells (16, 19). Adenosine phosphorylase activity, due to mycoplasmal contamination, could also convert adenine to adenosine, but the cells were shown to be mycoplasma free by two separate detection methods. One of these methods was specifically based on adenosine phosphorylase activity present in mycoplasma. Further, we never observed adenosine in any of the radiolabeled cellular fractions, even after 4 h of incubation. This result suggests that a purine nucleoside phosphorylase activity or a 5' nucleotidase activity was lacking in the cells we examined. It is also possible that adenine was metabolized to other purine forms (e.g., hypoxanthine or guanine) which were then converted to monophosphate forms via HPRT activity. This possibility is unlikely because HPRT activity is not required for expression of the APRT phenotype (Turker et al., in preparation). Therefore, we conclude that genuine adenine metabolism occurs in the APRT-deficient pseudorevertant cell lines, although more work will be necessary to determine whether such salvage initiates with the conversion of adenine to 5'-AMP.

High levels of adenine salvage in the pseudorevertant cell lines were clearly attributable to exposure to the antibiotics alanosine and azaserine. When the antibiotics are removed from these cells, their ability to salvage adenine falls rapidly, particularly for the cell line TT-17R21. Furthermore, when these cell lines are maintained in DAP medium and then transferred back to media containing adenine and one of the antibiotics, adenine salvage is rapidly restored. We were able to reproduce this result with the homozygous deficient parental lines TC-21 and TT-17 despite their being maintained in DAP for several months.

Azaserine blocks de novo purine biosynthesis and therefore inhibits 5'-IMP formation (7). Alanosine inhibits the conversion of 5'-IMP to adenylosuccinate (5) and thereby inhibits the formation of 5'-AMP (adenylsuccinate is directly converted to 5'-AMP). Since azaserine and alanosine are both capable of inducing adenine salvage, it is plausible to consider 5'-AMP starvation as the event which triggers this induction (Fig. 3). The effect of DAP on adenine salvage in the pseudorevertant cell lines is more difficult to assess. Removal from selective pressure (adenine plus antibiotic) is sufficient to cause a reduction in adenine salvage, and this drop is hastened by DAP only for TC-21R10 cells (Fig. 3). Further work will be necessary to clarify the relationship between adenine salvage and DAP in the pseudorevertant cell lines and to determine why this salvage mechanism is DAP^r.

Previously, we could not demonstrate a link between pseudoreversion and detectable APRT activity in cell extracts (Turker et al., in preparation). In this work, we demonstrated an APRT-like CRM in the pseudorevertant cell lines. This CRM was detected in pseudorevertants with a high level of adenine salvage, but reduced quantities were observed in the pseudorevertant lines maintained in DAP and in the homozygous deficient parental cell lines TC-21 and TT-17. Physical characterization of this APRT-like CRM will be necessary to determine its relationship, if any, to the wild-type APRT enzyme. The nature of the relationship between this CRM and adenine salvage is still an open question.

In conclusion, we would like to suggest that the results reported here are relevant for a more complete understanding of the regulation of adenine salvage in mammalian cells. It is commonly accepted that adenine salvage is not regulated in mammalian cells but instead is maintained at a high level via the constitutive expression of APRT. Consistent with this assumption is the recent observation that a G-C-rich region exists within 200 base pairs of the mouse APRT transcription start site. This region is similar to promotor regions observed for other housekeeping genes (8). This assumption, however, has been based on biochemical assays with selected cultured cell lines or with homogenized tissue extracts. We have described APRT-deficient cell lines with low constitutive levels of adenine salvage. This salvage can be induced to higher levels by adding inhibitors of de novo 5'-AMP synthesis, and it declines rapidly after removal of these inhibitors from the growth medium. Thus, our results indicate a novel second level of regulation for adenine salvage in mammalian cells. We hypothesize that this control of adenine salvage is sensitive to intracellular 5'-AMP levels and that it is normally masked by the high constitutive level of APRT expression. In support of this hypothesis, we note a second putative promotor (a 5' concensus promotor) approximately 700 base pairs 5' to the transcriptional start point of the mouse APRT gene (8). This hypothesis also implies that a *cis* regulatory locus is responsible for constitutive APRT synthesis in mammalian cells and that it is no longer functional in the pseudorevertant cell lines or in the homozygous deficient parental cells they were derived from. This may be the G-C-rich promotor described above.

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LITERATURE CITED

1. **Atkins, J. H., and S. M. Gartler.** 1968. Development of a nonselective technique for studying 2'6'diaminopurine resist-

ance in an established murine cell line. Genetics **60**:781–792.

2. **Bradley, W. E. C., and D. Letovanec.** 1982. High frequency nonrandom mutational event at the adenine phosphoribosyltransferase (APRT) locus in sib-selected CHO variant heterozygous for APRT. Somatic Cell Genet. **8**:51–66.
3. **Cartier, P. H.** 1980. Adenine uptake by isolated rat thymocytes. J. Biol. Chem. **255**:4574–4582.
4. **Chasin, L. A.** 1974. Mutations affecting adenine phosphoribosyltransferase activity in Chinese hamster cells. Cell **2**:37–41.
5. **Chu, E. H. Y., and S. S. Powell.** 1976. Selective systems in somatic cell genetics. Adv. Hum. Genet. **7**:189–258.
6. **Cowell, J. K.** 1982. Double minutes and homogeneous staining regions: gene amplification in mammalian cells. Annu. Rev. Genet. **16**:21–59.
7. **Demars, R.** 1974. Resistance of cultured human fibroblasts and other cells to purine and pyrimidine analogues in relation to mutagenesis. Mutat. Res. **24**:335–364.
8. **Dush, M. K., J. M. Sikela, S. A. Khan, J. A. Tischfield, and P. J. Stambrook.** 1985. Nucleotide sequence and organization of mouse APRT gene: presence of a coding region common to animal and bacterial phosphoribosyltransferase that has a variable intron/exon arrangement. Proc. Natl. Acad. Sci. USA **82**:2731–2735.
9. **Fuscoe, J. C., R. G. Fenwick, Jr., D. H. Ledbetter, and C. T. Caskey.** 1983. Deletion and amplification of the HGPRT locus in Chinese hamster cells. Mol. Cell. Biol. **3**:1086–1096.
10. **Hamet, M., C. Bonissol, and P. Cartier.** 1980. Enzymatic activities on purine and pyrimidine metabolism in nine mycoplasma species contaminating cell cultures. Clin. Chim. Acta **103**:15–22.
11. **Kozak, C., E. Nichols, and F. H. Ruddle.** 1975. Gene linkage analysis in the mouse by somatic cell hybridization: assignment of adenine phosphoribosyltransferase to chromosome 8 and alpha-galactosidase to the X chromosome. Somatic Cell Genet. **1**:371–382.
12. **Lederberg, J., and E. M. Lederberg.** 1952. Replica plating and indirect selection of bacterial mutants. J. Bacteriol. **63**:399–406.
13. **Linder, S., A. W. Coleman, and J. M. Eisenstadt.** 1984. Amplification of hypoxanthine-guanine phosphoribosyltransferase genes in chromosome-mediated gene transferents. Mol. Cell. Biol. **4**:618–624.
14. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
15. **Luria, S., and M. Delbruck.** 1943. Mutations of bacteria from virus sensitivity to virus resistance. Genetics **28**:491–511.
16. **Martin, D. W., Jr.** 1983. Metabolism of purine and pyrimidine nucleotides, p. 342–359. *In* D. W. Martin, Jr., P. A. Mayes, and V. W. Lange (ed.), Harper's review of biochemistry. Lange Medical Publications, Los Altos, Calif.
17. **McGarrity, G. R., and D. A. Carson.** 1982. Adenosine phosphorylase-mediated nucleoside toxicity: application towards the detection of mycoplasmal infection in mammalian cell cultures. Exp. Cell Res. **139**:199–205.
18. **Melton, D. W., D. S. Konecki, D. H. Ledbetter, J. F. Hejtmancik, and C. T. Caskey.** 1981. In vitro translation of hypoxanthine/guanine phosphoriboxyltransferase mRNA: characterization of a mouse neuroblastoma cell line that has elevated levels of hypoxanthine/guanine phosphoribosyltransferase protein. Proc. Natl. Acad. Sci. USA **78**:6977–6980.
19. **Murray, A. W.** 1971. The biological significance of purine salvage. Annu. Rev. Biochem. **40**:811–826.
20. **Nalbantoglu, J., O. Goncalves, and M. Meuth.** 1983. Structure of mutant alleles at the APRT locus of chinese hamster ovary cells. J. Mol. Biol. **167**:575–594.
21. **Russell, W. C., C. Newman, and D. H. Williamson.** 1975. A simple cytochemical technique for demonstration of DNA in cells infected with mycoplasmas and viruses. Nature (London) **253**:461–462.
22. **Simon, A. E., M. W. Taylor, and W. E. C. Bradley.** 1983. Mechanism of mutation at the *aprt* locus in Chinese hamster ovary cells: analysis of heterozygotes and hemizyogtes. Mol. Cell. Biol. **3**:1703–1710.
23. **Simon, A. E., M. W. Taylor, W. E. C. Bradley and L. H. Thomson.** 1982. Model involving gene inactivation in the generation of autosomal recessive mutants in mammalian cells in culture. Mol. Cell. Biol. **2**:1126–1133.
24. **Siminovitch, L.** 1976. On the nature of heritable variation in cultured somatic cells. Cell **7**:1–11.
25. **Turker, M. S., A. C. Smith, and G. M. Martin.** 1984. High frequency "switching" at the adenine phosphoribosyltransferase locus in multipotent mouse teratocarcinoma stem cells. Somatic Cell Mol. Genet. **10**:55–69.
26. **Witney, F. R., and M. W. Taylor.** 1978. Role of adenine phosphoribosyltransferase in adenine uptake in wild-type and $APRT^-$ mutants of CHO cells. Biochem. Genet. **16**:917–926.
27. **Zownir, O., J. C. Fuscoe, R. Fenwick, and J. Morrow.** 1984. Gene amplification as a mechanism of reversion at the HPRT locus in V79 Chinese hamster cells. J. Cell. Physiol. **119**: 341–348.